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EDITORIAL REVIEW

Proton translocating ATPases: Issues in structure and function

Several lines of evidence indicate that distal nephron acidification occurs by means of a primary electrogenic, proton translocating event. This subject has recently been reviewed extensively [1]. However, certain key observations serve as an introduction to a description of the final effector of distal nephron acidification, a proton translocating ATPase. Although it was observed by Pitts and Alexander that some degree of functional Na/H^+ exchange occurs in the distal nephron [2], it is apparent that a transporter-coupled exchange of the sort that mediates proximal tubular acidification cannot be responsible for collecting duct acidification; given that the kidney is capable of generating a 3 pH unit, lumen to blood gradient, utilization of sodium as the driving force for this gradient, assuming a 1:1 Na/H^+ coupling ratio, would require that the lumenal Na gradient be 1 M, even if intracellular [Na] were 1 mM. The predicted sodium independency of this acidification event was demonstrated in studies conducted in turtle urinary bladder [3], and in rabbit cortical [4] and medullary [5] collecting duct. In all instances, urinary acidification was found to proceed by means of a primary electrogenic event which is directly modulated by aldosterone. Controversy regarding two possible acidification mechanisms, proton secretion versus bicarbonate reabsorption, was resolved by the demonstration that rat distal nephron acidification is accompanied by the generation of an acid disequilibrium pH [6]. Such a finding provided strong evidence that acidification proceeds by means of proton secretion. The first evidence for the molecular mechanism of this event was provided in studies by Dixon and Al-Awqati [7], who demonstrated that imposition of an adverse, electrochemical proton gradient across isolated turtle urinary bladder epithelium resulted in an increase in intracellular ATP stores, indicating that turtle bladder (and by analogy, renal collecting duct) acidification occurred by means of an electrogenic, reversible proton translocating ATPase. In extension of these studies, it was found that apical membrane vesicles prepared from turtle urinary bladder contained an electrogenic, non-mitochondrial proton translocating ATPase [8], which bore a close similarity to a proton translocating ATPase previously localized to lysosomes [9].

Simultaneous advancement of knowledge of the distal nephron acidification process subsequently occurred in two disciplines with an outlining of the cellular physiology of proton pump insertion and with biochemical definition of the nature of the proton pump itself, derived from examination of the molecular characteristics of related proton pumps localized to an extraordinarily wide variety of intracellular organelles.

With respect to the cellular mechanism of acidification, it has been demonstrated functionally [10] and morphologically [11] that distal nephron and turtle urinary bladder acidification is regulated, in part, through endocytotic and exocytotic events in the mitochondrial rich cells. Evidence has accrued that a subapical collection of acidic organelles (tubulo-vesicular bodies) serve as a mobile repository for proton pumps, which can be inserted or removed from the apical surface of acidifying cells as a function of acid-base perturbations. Details of this process are elaborated in a recent review [12]. In addition, electron micrographic studies demonstrated the presence of apical membrane and tubulo-vesicular body "studs" [13] and "rod-shaped particles" [14] which appear to correlate with the functional degree of acidification occurring within the urinary epithelial systems.

Coincident has been an emerging literature of the molecular characterization of the proton pump. It has rapidly become apparent, as shown in Table 1, that a wide variety of intracellular organelles [15] contain proton translocating ATPases which share striking similarities with the proton pump presumed responsible for distal nephron acidification [16, 17]. Coordinate with this widespread distribution of proton pumps is a marked diversity of function. In some instances, the physiologic role of the pump is well described (such as, lysosomes and endosomes), whereas in other instances (endoplasmic reticulum) the functional importance of the proton pump awaits definition [15, 18].

It is the purpose of this review to detail the known characteristics of this new class of proton translocating ATPases with particular emphasis upon specific issues relevant to the role of the proton pump in urinary acidification.

Taxonomy of proton pumps

In general, proton pumps which are directly energized can be subdivided into three classes.

First, and best characterized, are the light-energized, electrogenic proton pumps exemplified by bacteriorhodopsin which has been shown to be composed of a single polypeptide with a molecular mass of 27 kDa. X-ray crystallographic analysis reveals that the pump consists of seven transmembranous, α helical coils [19]. When energized by light the enzyme enters a catalytic cycle, composed of a controversial number of discrete steps, culminating in the vectoral transmission of a proton at a calculated stoichiometry of 2 protons/photon [20].

A second class of proton pumps, the redox variety, are exemplified by the cytochrome (oxidative) chain of bacteria, chloroplasts and mitochondria. In the latter case, the chain is composed of redox pumps at sites I, II, and III [21], as well as cytochrome oxidase, which has recently been shown to be a redox pump [22]. Considerable controversy exists as to the subunit composition of the latter, and the difficulty in defining polypeptide composition is instructive and pertinent to the proton pump of focus in this discussion. It has been shown that

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Table 1. Organelles to which vanadate and oligomycin-resistant, N-ethylmaleimide-sensitive proton pumps have been localized

Mammalian sources	Plant sources
Lysosomes	Tonoplasts of beet, corn and oat
Clathrin-coated vesicles	
Endosomes	
Chromaffin granules	
Golgi membranes	Fungal sources
Endoplasmic reticulum	Tonoplast of *Neurospora*
Platelet dense granules	
Synaptic vesicles	
Leukocyte tertiary granules	
Pituitary neurosecretory granules	

A functional overview is in [15, 18].

there are some 12 to 13 polypeptides in cytochrome oxidase preparations which purify in a stoichiometric manner with the copper and heme components of the enzyme [23]. Are these polypeptides subunits? The answer is in part semantic. If one chooses a functional definition, that is, what is the minimal polypeptide composition required for proton pumping, catalyzed by the reconstituted, purified system, then the answer is six [24] for mammalian systems, and two for the bacterial variety [25]. It can be counter argued that the remaining polypeptides are indeed subunits, but serve the function of coordinating membrane orientation and subunit assembly during in vivo biogenesis, services which are rendered unnecessary by the artifact of integral membrane protein reconstitution.

The third group of directly energized proton pumps utilize ATP as an energy source. Two types of these ATPases have been well characterized, the E_1-E_2 type enzymes and the F_1F_0 type proton pumps. The former is exemplified by the gastric mucosa [26, 27] and *Neurospora* plasma membrane [28] proton translocating ATPases. Like all E_1-E_2 type enzymes (which include Na^+-K^+ ATPase and the Ca^{++} ATPases), these proton pumps have as part of their catalytic cycle an acyl phosphate intermediate [29]. Vanadate (VO_4) serves as an inhibitor of these pumps by substituting for phosphate during the catalytic cycle; typically micromolar amounts of VO_4 are needed for this inhibition. Structurally, these are simple systems as exemplified by the *Neurospora* plasma membrane proton pump, which is composed of a single polypeptide of molecular mass of 105 kDa [30].

More complicated is the second type of proton ATPases, those of the F_1F_0 variety. These pumps are localized in mitochondria, chloroplasts and bacterial membranes, where they serve a primary function of *synthesizing* ATP. A detailed description of these pumps is beyond the scope of this review, but several features are particularly important. Structurally, these are large complexes of about 350 kDa. The mitochondrial synthetase is composed of two functional units, the ATP hydrolytic/synthetic component (F_1) and a hydrophobic transmembranous sector which serves as a proton channel (F_0). F_1 is itself composed of five subunits and F_0 is composed of an additional three subunits. The F_1 component is a peripheral membrane protein, which does not require detergents for solubilization, in contrast to F_0 [31]. Within the mitochondria, protons, transported by the parallel oxidative chain, are driven through the proton pore, and the energy of the transmembranous proton gradient ($\Delta\mu H^+$) is thus utilized by F_1 through an unknown mechanism to energize ATP synthesis, as originally proposed by Mitchell [32] and demonstrated by Kagawa and Racker [31]. Perhaps in accord with its role as an ATP synthesizer, the mitochondrial F_1F_0 ATPase catalyzes a relatively high rate of $^{32}P_i$-ATP exchange, a process which requires a facile conversion between ATP synthetic and hydrolytic modes [33]. Unlike the E_1E_2 type enzymes, all F_1F_0 type pumps are vanadate resistant and lack a discernable phosphoenzyme intermediate. Mitochondrial F_1F_0 is inhibited by two highly-specific inhibitors, mitochondrial ATPase inhibitor [34] (which some claim to be a subunit) and efrapeptin [35]. Less specific inhibitors include azide, oligomycin and DCCD. When separated from F_0, F_1 retains its sensitivity to all of the above inhibitors except oligomycin and DCCD. These two inhibitors bind to F_0 ($Factor_{oligomycin}$, *not* $Factor_{zero}$), and it has been clearly shown that proton conduction through isolated and reconstituted F_0 is inhibited both by DCCD [36] and oligomycin [31]. Inhibition of ATPase activity of the intact F_1F_0 complex by DCCD and oligomycin can thus be attributed to the sequential inhibition of proton conduction, and subsequently ATP hydrolysis in the tightly coupled system. Finally, the F_1F_0 complex is inhibited by a wide variety of nonselective inhibitors, including SITS, phenothiazines, DIDS, quercetin, and Dio 9, a compound of unknown composition and known nonspecificity.

At this point, two quotes seem appropriate: "Only uninhibited investigators use inhibitors" (Cori), and "If you accept the statement that only uninhibited investigators use inhibitors, you will soon find out what kind of people work in the field of oxidative phosphorylation" (Racker).

Recently, a third group of proton translocating ATPases (Table 1) have been described which have resistance to both the E_1E_2 type inhibitor, VO_4, and to the F_1F_0 type inhibitors, efrapeptin and oligomycin. In contradistinction, these pumps are inhibited by the water-soluble sulfhydryl reactive reagent, N ethylmaleimide (NEM), to which the mitochondrial pump is resistant [15, 16, 18]. Thus, based upon a crude taxonomy of inhibitor sensitivities, it would appear that this new class of proton pumps is unrelated to the E_1E_2 and F_1F_0 type pumps.

Molecular and functional characteristics of the proton pumps

Before going the way of workers in the field of oxidative phosphorylation, available knowledge of the structure of this new class of proton pumps warrants review. Recently, a number of ATPases have been isolated from proton pump containing endomembranes [37–43], as shown in Table 2. In large part these isolated ATPases have an inhibitor sensitivity profile which closely matches that of the proton pump present in the membranes from which they were isolated, thus suggesting that they are, indeed, proton pump related. Several features are notable. First, when it has been determined, it is apparent that the intact complexes are large, with holoenzyme molecular mass estimates ranging from 300 to 600 kDa. Second, there is remarkable consistency in the polypeptide composition of the isolates. Notable is the uniform conservation of the 67 to 80 kDa, 57 to 64 kDa and 15 to 19.5 kDa polypeptides. Several key questions emerge from examination of the features of these ATPase isolates: Are the polypeptides present in these preparations genuine subunits? If so, what are the roles of the subunits? Do intrinsic, subtle differences exist amongst these pumps, particularly those of mammalian origin, which might

Table 2. Estimated molecular mass of putative subunits and of holoenzyme of isolated H^+-ATPases

Membrane	Plant Tonoplast			Fungal tonoplast		Chromaffin granules		Clathrin coated vesicles
						Bovine		
Source	Beet	Corn	Oat	Neurospora	Yeast	Adrenal	medulla	Bovine brain
Reference	37	38	39	38	40	41	42	43
Polypeptides					kDa			
						140		
							115[a,b,c]	116[a]
	67	72	72[a,b]	70[a,b]	80[a,b]	70	72	70[a,b]
	57[a]	62	60	62	64	57	57	58
						41		40
							39[b]	38
								34
						33		33
	16[c]		16[c]	15[c]	19.5[c]	16[c]	16[c]	17[c]
Holoenzyme *kDa*		300–500	300–600	520				530
Reconstitutively active	No	No	No	No	No	No	No	Yes

[a] Nucleotide-binding
[b] NEM-binding
[c] DCCD-binding

account for targeting to disparate organelles or diversification in function and differential regulation? What is the final decision regarding the relationship of these pumps to classic F_1F_0 type ATPases? Incomplete answers to these questions are presented below.

Nature of subunit composition and function

As noted in reference to cytochrome oxidase, precise definition of subunit composition of a multimeric enzyme is intrinsically difficult. Because elucidation of subunits required for in vivo biogenesis of this system is in the distant future, and because it is our bias, we will focus upon functional definition of subunit composition, that is, what are the minimal polypeptide requirements for reconstitutively-active proton pumping? The most suspicious preparation with respect to the possibility of contamination by polypeptides is the proton pump of bovine brain clathrin-coated vesicles, where eight polypeptides are found in the final isolate [43]. The fact that similar enzymes, isolated from the same species [41, 42], have fewer polypeptides would indicate, barring intrinsic differences among the pumps, that fewer polypeptides are absolutely required for ATP hydrolysis. It should be pointed out, however, that the clathrin-coated vesicle, proton translocating complex is the only purified enzyme which has been demonstrated to catalyze proteoliposome acidification when reconstituted, and thus it is possible that in other instances, critical subunits necessary for proton pumping have been removed during purification procedures. An analogy, described above, exists with F_1F_0 type proton pumps in which F_1, when isolated separate from F_0, is capable of hydrolyzing ATP, but not pumping protons. This issue of final definition of subunit function through determination of the minimal polypeptide requirements for reconstitutively-active proton pumping is likely to require years of work, yet such an analysis of mitochondrial F_1 eventually led both to a precise description of subunit composition, as well as function [44].

What can be said at present of subunit function? The facts are that in most instances the ≅70 kDa subunit can be photoaffinity labeled with ATP or derivatized ATP, and that in two bovine preparations (chromaffin granule [42] and clathrin-coated vesicles [43, 45]) an additional nucleotide binding site exists in the ≅116 kDa polypeptide. There is a growing speculation among researchers working in plant and fungal systems [38–40] that the 70 kDa polypeptide is the ATP hydrolytic subunit. The situation is less clear in the mammalian systems, and Cidon and Nelson [42] have proposed that the 115 kDa subunit of the chromaffin granule pump is the ATP hydrolytic subunit of his system. We have purified from bovine brain clathrin-coated vesicles a 116 kDa ATPase which shares an extraordinary number of similarities with ATPase activity of the 530 kDa holoenzyme, which further supports this notion [46]; in such a case, the 70 kDa subunit could serve as a nucleotide regulatory subunit. This is supported by the observations that the clathrin-coated vesicle proton pump is highly sensitive to ambient [ADP] [47], whereas the 116 kDa ATPase is not. Pending molecular reconstitution of the 116 kDa ATPase to a 116 kDa polypeptide-depleted preparation, the role of this peptide remains an open question. Further complicating the issue are reports that an NEM-sensitive ATPase, isolated from bovine kidney medulla, lacks any subunit of molecular mass greater than 70 kDa [48]. Given the relative consistency in nucleotide binding, the apparent phylogenetic preservation, and the frequent finding of NEM binding, it appears likely that the 70 kDa polypeptide is a true subunit and is directly or indirectly linked to the ATP hydrolytic function of the enzyme. However, it is important to note that ATP binding cannot be equated with function, and definition of the ATP hydrolytic subunit(s) awaits further resolution.

Likewise, the ≅16 kDa polypeptide of these proton pumps which is uniformly labeled with [^{14}C]DCCD indicates strongly that this is indeed a true pump subunit. The finding of DCCD sensitivity of the pump, as well as the labeling of a low molecular weight polypeptide has led to widespread speculation that this component, analogous to F_0, serves as a transmembranous proton pore [37–43]. Support for the presence of such a pore as part of the clathrin-coated vesicle proton pump has

been presented where it has been shown that the partially purified ATPase, which is not reconstitutively active, can be reassembled to ATPase-depleted membranes to restore proton pumping activity [49]. Again, proof that the 17 kDa subunit has such channel function requires molecular reconstitution of isolated pump components, although it is safe to say that the finding that the DCCD binding polypeptide is separate from the NEM and ATP binding subunits strongly supports this notion.

The role of the highly preserved ≅58 kDa subunit remains wholly conjectural, but by analogy to F_1F_0 type pumps [31], this may serve as a coupling factor which functions to link the energy transformer to the transmembranous proton sector.

Intrinsic differences among proton pumps

As shown in Table 2, apparent differences in candidate subunits are present in various mammalian proton pump preparations; however, no group of investigators can claim purity at present and in addition, comparisons of molecular mass determination of polypeptides from polyacrylamide gel electrophoresis performed in different laboratories under different conditions is not a convincing means of determining if real differences do exist. There are three lines of evidence at present that intrinsic differences do exist. First, in side by side comparison, it has been determined that ≅70 kDa subunits of the bovine adrenal chromaffin granule and clathrin-coated vesicle proton pumps have about a 3 kDa molecular mass difference [50]. Unfortunately, interpretation is rendered difficult because of the possibilities of either proteolysis in the preparations or differences in glycosylation of the subunit. Second, it has been shown that certain proton ATPases (lysosomal and endosomal) can utilize GTP as substrate, whereas the clathrin-coated vesicle and renal medulla proton pumps have a strict requirement for ATP. Again, interpretation is difficult, given the possibilities of preparations being contaminated with nucleoside diphosphokinase and commercial sources of nucleotide being impure [47]. Third, and most convincing, analysis of Chinese hamster ovarian (CHO) cell lines which bear a temperature-sensitive, conditionally lethal mutation in vacuolar acidification indicates that structural and functional differences between the endosomal and lysosomal pump do exist [51]. One such cell line, G.7.1, has been shown to have a mutation which imparts thermolability to isolated endosomal, but not lysosomal, acidification [52], and available evidence indicates that the mutation is expressed at the level of the proton pump itself [53]. Nonetheless, the issue of intrinsic differences among proton pumps from various organelles of the same mammalian species remains an open, critical question, and resolution of this issue may well bring to bear novel insight into cellular protein sorting events, should intrinsic differences exist. This issue is most likely to be definitively resolved through a molecular biological approach, in conjunction with mutant cell line analysis.

Relationship to classic F_1F_0-type pumps

The most likely interpretation of our present state of information regarding this new class of proton pumps is that they are members of the F_1F_0 family. In this regard, it has been proposed that both "classic" F_1F_0 pumps and these "new" F_1F_0 pumps share a common ancestry in the primordial proton pump of anaerobic bacteria [54], although the results of the ultimate taste test (sequence homology) are still pending. It appears, if this hypothesis is correct, that the divergence has been extreme. The anaerobic bacteria proton pump can function both to synthesize and hydrolyze ATP, the mitochondrial pump functions in a dedicated synthase mode, and the new F_1F_0 pumps appear to function exclusively as ATPases, as is suggested by the relatively low rate of $^{32}P_i$-ATP exchange characteristic of the clathrin-coated vesicle proton pump [55]. Whether the directionalities of these pumps are extrinsic or intrinsic features of these physiologic systems awaits direct experimentation. Certainly, mitochondrial F_1 synthesizes ATP because of energization via the oxidative chain, and in the absence of such a parallel proton pump, endomembrane proton ATPases cannot, of course, autoenergize ATP synthesis. Alternatively, the endomembrane pumps, as compared with F_1, may be less reversible for any given degree of energization. In addition to the aforementioned inhibitor sensitivity and directional differences among the two F_1F_0 type systems, it appears that major structural changes have occurred. Specifically, mitochondrial F_1 is a peripheral membrane protein, whereas the holoenzyme of the new class of pumps evidently requires rather harsh detergent treatment for extraction. Moreover, the F_1 ATPase, when separated from F_0, has no phospholipid requirements, whereas both the chromaffin granule [42] and clathrin-coated vesicle proton pumps [43] as well as the 116 kDa ATPase [46] of clathrin-coated vesicles require phosphatidyl serine for activation and stabilization of enzyme function. Simply put, the delipidated enzymes become dilapidated, which is hardly an expected feature of a peripheral membrane protein.

Proton pumps and the kidney

Having reviewed the known characteristics of these new proton pumps, it is appropriate to relate the findings to the kidney. As noted in the introductory comments, all available evidence indicates that distal nephron acidification is effected by an electrogenic proton translocating ATPase. In light of the widespread intracellular organelle distribution of the new class of proton ATPases, it however remains a possibility that the proton pumps attributed to renal medulla plasma membrane, and turtle urinary bladder vesicle preparations represent a contamination of the final "apical" membrane preparations with endomembranes of other sources which contain proton pumps.

This possibility increases when the technical aspects of membrane vesicle preparation from renal medulla are considered. Unlike the isolation of native organelles, isolation of plasma membrane vesicles requires the fragmentation and reassembly of the plasma membrane bilayer. Such a maneuver allows for membrane hybridization events and is possible that non-plasma membrane bilayers might "intercalate" with those of the true cell membrane. As a control, such plasma membrane preparations are routinely analyzed for the presence of organelle-specific marker enzymes. This is hardly definitive, and indeed such an approach led to the erroneous conclusion that clathrin-coated vesicles contain a Ca^{++} translocating ATPase [56]. The situation is made even more difficult because of the complex architecture of the kidney. Ignoring the abundant interstitial cells, a variety of nephron segments course through the renal medulla, and indeed, in rabbit, there are six to seven pars rectae, thin descending limbs of Henle, and thick ascending limbs of Henle for every collecting duct. To definitely prove

that a vesicle enrichment results in a homogenous population of membranes which are solely derived from one side of one cell type is extraordinarily difficult. Reflecting this problem is the fact that there are now several "plasma membrane" preparation techniques from kidney medulla which share very little in common, and indeed, almost all of these preparations have NaK ATPase activities [16, 17, 57, 58]. Two further complications arise. NaK ATPase has recently been localized to intracellular membrane compartments [59], indicating that what we routinely regard as definitive markers for a given system may be suspect. Moreover, evidence has been presented that in HCO_3 secreting cells of the kidney that "the" proton pump is placed on the basolateral membrane [12]. In simplest terms, there is at present, no highly reproducible, reliable preparation of apical membranes from acidifying epithelial cells, and localization of a proton pump to the surface of the mitochondrial rich cell by such a method is only indirect evidence in support of enzyme localization.

The obvious approach to the resolution of this problem lies in immunocytochemical analysis of proton pump distribution in the kidney. Indeed, two such studies have been reported in a preliminary form where antiproton pump antibodies reveal staining at the base of the brush border of proximal tubular cells, within thick ascending limb cells, and at more comforting locations: apical and basolateral membranes of selected cortical collecting duct cells and apical membrane of medullary collecting duct cells [60, 61]. In addition, staining of intracellular vacuoles have also been noted. With respect to the proximal tubular cell staining, mounting evidence indicates that proximal tubular acidification cannot wholly be attributed to the described Na/H^+ antiporter, and indeed, up to 35% of bicarbonate reabsorption can be attributed to a non-Na dependent mechanism, perhaps a proton pump [62]. Stainings within thick ascending limb cells, where there is no physiologic evidence to underwrite the notion that such a proton pump exists, is more difficult to explain. However, at present it is not clear whether the stainings of thick ascending limb cells are intracellular (perhaps reflecting dense endomembrane concentration) or truly localized to the cell surface. If intrinsic differences among this new class of proton pumps do exist, then it may be possible in the future to clarify more precisely the distribution of pumps through the use of proton-pump subclass-specific antibodies.

An alternative approach has been attempted to aid in localization of the proton pump along the nephron, namely a microenzymologic analysis of H^+ ATPase activity in individually dissected segments. In such studies, it has been found that ouabain-, azide-, and vanadate-resistant, NEM-sensitive ATPase activities are found in rank order in proximal convoluted tubules, thick ascending limb and collecting duct [58]. The results of these studies are thus consistent with the described immunocytochemical findings of nephron proton pump localization. Unfortunately, such analysis cannot discriminate between plasma membrane H^+ ATPase activity and that of endomembranes. Moreover, NEM is a very nonspecific inhibitor and it is simply not possible to equate NEM-sensitive ATPase activity with proton pump, relevant ATPase activity in any more than a loose qualitative fashion. To date, there is no specific inhibitor of these new proton ATPases, as is the case with Na^+K^+ ATPase (ouabain) and mitochondrial F_1F_0 (efrapeptin). Clearly, identification of such an inhibitor, if plasma membrane, proton pump specific, would be enormously useful in determining the relationship between alterations in collecting duct (and proximal tubular) acidification rates and H^+ ATPase activity.

Lastly, it should be reemphasized that it is possible that more than one type of proton pump may modulate urinary acidification, perhaps in a segment specific fashion. In this regard, the role of an apparent HCO_3^- stimulated ATPase [63] awaits clarification, and indeed it has not been determined if the renal proton pump of the new F_1F_0 class is HCO_3^- activated. More certain is the lack of importance of Na^+K^+ ATPase as a physiologically-important proton pump. While purified, reconstituted NaK ATPase can catalyze low rates of proton pumping, the observations that the presence of 3.3 mM Na inhibits this process and that the pH optimum is 5.6, with virtually no proton pumping occurring above 6.0 place this finding in an area of interest for bioenergeticists, and not physiologists [64].

Insights into renal proton pump regulation

Before entering into this discussion, it should be made clear that the bioregulation of renal proton pump function is poorly understood. We have attempted to draw, where possible, from current knowledge of the biochemical properties of other members of this new class of efrapeptin-resistant, NEM-sensitive proton pumps. Extrapolation from our own system, the bovine brain clathrin-coated vesicle proton pump, requires clarification. We have chosen these organelles, which serve as a ubiquitous mechanism by which endocytosis, exocytosis and protein targeting is mediated [65] because their presence in every nucleated eukaryotic cell gives credence to a widespread applicability of the pump characteristics and because the specific activity of the proton pump within these vesicles is quite high.

It is clear that clathrin-coated vesicles contain a proton pump, based upon organelle isolations performed in three laboratories [55, 66, 67], immunoprecipitation of proton pumping activity by anticlathrin antibodies [55] and immunocytochemical analysis indicating that some (but not all) coated vesicles are acidic [68]. It is also clear that the tubular vesicular bodies are not clathrin-coated vesicles [69], and the possibility exists that the proton pump responsible for urinary acidification is unique and is simply shuttled to tubulovesicular bodies by the coated vesicles, which contain a pleuripotent pump designed to carry out constitutive housekeeping functions. At present, however, there are no discernable functional differences between the bovine brain clathrin-coated vesicle and renal medulla proton pumps [16] and we speculate that if polypeptide differences do occur, they are likely related to individualization of the pumps with "zip codes" which direct delivery.

As noted in the introduction, an important mechanism by which collecting duct and turtle bladder acidification is regulated has been defined by description of acidic tubular vesicular body/plasma membrane fusion events in acidifying cells [12]. Almost certainly other levels of regulation exist, and chloride is a likely candidate. It has been shown that rabbit, medullary collecting duct acidification proceeds by means of electrogenic, acidifying process and that chloride modulates the process through two mechanisms: first by facilitating the exit of cellular HCO_3 which accrues as a function of proton extrusion; this occurs by means of a basolateral membrane, SITS sensitive,

chloride/HCO_3 exchanger. Second, chloride serves as the dominant co-ion, counterbalancing electrogenic acidification in maintenance of net electroneutrality [70]. A third, direct effect of chloride upon modulation of medullary collecting duct acidification is suggested by the finding that several of these new proton ATPases from plant sources are directly chloride activated [71]. Evidence indicates that chloride does indeed directly activate both the purified, reconstituted clathrin-coated vesicle proton pump [72] and the microsomal renal medulla proton pump [73] in a co-ion independent fashion, as well as an ATPase of renal medulla vesicles which is sensitive to an inhibitory, antiproton pump antibody. Maximal stimulation of proton pumping and renal medulla vesicle ATPase activity occurred at a [Cl] of 20 mM, with an apparent Km of about 5 mM [72]. This suggests that alterations in extracellular Cl could be transmitted to effect a change in intracellular Cl through the basolateral Cl/HCO_3 exchanger, or chloride channel [74], and the apical membrane pump could thus be activated or repressed as a function of chloride activity. Thus in a setting of hyperchloremic metabolic acidosis, intracellular [Cl] might rise and thus stimulate the apical membrane pump to facilitate correction of the systemic disorder. Consonant with this hypothesis are the findings in isolated perfused tubule studies that medullary collecting duct acidification is indeed stimulated by bath composition mimicking that of such an acid base derangement [75], and that intracellular chloride activity, as measured in distal tubules of *Amphiuma*, ranges from 7 to 21 mM [76], a basal activity well within the range such that slight changes would be predicated to modulate pump function.

The issue of chloride regulation of cortical and medullary collecting duct acidification warrants further evaluation at the level of the tubulovesicular body/plasma membrane fusion event which occurs in proton (and HCO_3) secreting cells. As described, the subapical membrane vesicles of these cells are acidic [10], and that acidification is, evidently, affected by means of an electrogenic proton translocating ATPase. Any manifestation of $\Delta\mu H^+$ as an electrical potential is counterproductive to acidification, and the fact that acidification does occur in these subapical vesicles indicates that co-ion or counterion movement must occur. Supportive of the notion that Cl serves as the critical co-ion is evidenced from a variety of endomembrane vesicle studies that indicate that the proton pump operates in parallel with a chloride conductance [47, 77]. In one instance, it has been shown that the chloride conductance is a DIDS [47] and duramycin [78] inhibitable, electrogenic Cl^- transporter. One would expect that with fusion of the proton pump with the apical membrane that a chloride conductance would be expressed. Such, however, is not the case, as indicated by electrophysiologic studies in rabbit, medullary collecting duct [74], suggesting that the putative chloride transporter has been deactivated during or after fusion of the tubulovesicular body population with apical membranes. This in turn implicates the possibility of direct, molecular interaction of the Cl^- transporter with the proton pump through an allosteric mechanism. Although it has been demonstrated that the chloride transporter and proton pump of clathrin-coated vesicle can be functionally (and structurally) dissociated, the definition of interplay between the two transporters awaits purification of the Cl^- transporter.

A second possibility of pump regulation exists at the level of the lipid bilayer composition of the apical membrane of acidifying cells. It has been well described that aldosterone is a direct and potent modulator of both turtle urinary bladder and rabbit cortical and medullary collecting duct acidification [3–5]. In isolation of the proton translocating complex of clathrin-coated vesicles and chromaffin granules, it was determined that phosphatidylserine is a potent activator of the pump in a solubilized state [43]. Moreover, it is clear that minute alterations in the lipid composition of pure lipid liposomes into which the isolated CCV pump is reconstituted significantly alters proton pump activity [79]. As mineralocorticoids have been shown previously to alter the lipid composition of plasma membrane in target epithelia [80], the possibility remains open that the lipid bilayer composition of apical membrane may serve to activate or decrease proton pump turnover rates.

Conclusions

In this general overview of a new class of electrogenic proton translocating ATPases, assembled evidence indicates that these proton pumps are distant ancestors of classic F_1F_0 type ATPases. As pointed out, definition of the precise mechanisms by which these proton pumps in general, and the renal proton pump(s) in particular operate and are regulated awaits considerable work. Resolution of certain key issues, notably endomembrane H^+ ATPase microheterogeneity, molecular structure and cellular regulation are likely to result in implications pertinent not only to renal physiology, but also to basic issues in cellular and molecular biology.

Notes added in proof

In the past year, two reports have appeard on the reconstitution of proton pumping catalyzed by partially purified endomembrane proton pumps. The bovine adrenal chromaffin granule ATPase, which is composed of a minimum of 5 polypeptides, has been reconstituted without exogenous lipids (MORIYAMA Y, NELSON N: The purified ATPase from chromaffin granule membrane is an anion dependent proton pump. *J Biol Chem* 262:9175–9180, 1987). Also, an ATPase isolated from bovine kidney microsomes has been reconstituted using an enzyme preparation composed of 15 to 20 polypeptides (GLUCK S, CALDWELL J: Immunoaffinity purification and characterization of vacuolar H^+ ATPase from bovine kidney. *J Biol Chem* 262:15780–15789, 1987).

In addition, the role of the 17-kDa polypeptide of the clathrin-coated vesicle proton translocating complex has been functionally defined. When isolated from the holoenzyme, this subunit can be reconstituted and shown to serve as a DCCD-sensitive, transmembranous proton channel (SUN S-Z, XIE X-S, STONE DK: Isolation and reconstitution of the dicyclohexylcarbodiimide-sensitive proton pore of the clathrin-coated vesicle proton translocating complex. *J Biol Chem* 262:14790–14794, 1987).

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